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FimH-mediated autoaggregation of *Escherichia coli*

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Summary

Autoaggregation is a phenomenon thought to contribute to colonization of mammalian hosts by pathogenic bacteria. Type 1 fimbriae are surface organelles of *Escherichia coli* that mediate D-mannose-sensitive binding to various host surfaces. This binding is conferred by the minor fimbrial component FimH. In this study, we have used random mutagenesis to identify variants of the FimH adhesin that confer the ability of *E. coli* to autoaggregate and settle from liquid cultures. Three separate autoaggregating clones were identified, all of which contained multiple amino acid changes located within the N-terminal receptor-binding domain of FimH. Autoaggregation could not be inhibited by mannose, but was inhibited by growth at temperatures at or below 30°C. Using green fluorescent protein (GFP) as a reporter, we show that the autoaggregating clones do not mix with wild-type fimbriated cells. Electron microscopy shows that autoaggregating cells produce fimbriae with a twisted and entangled appearance. We present evidence that autoaggregating versions of FimH also occur in nature. Our results stress the highly adaptive nature of the ubiquitous FimH adhesin.

Introduction

Some bacteria are able to autoaggregate, a phenomenon readily observed in the microscope as a characteristic clumping of cells and, macroscopically, as flocculation and settling of cells from static liquid suspensions. A number of surface factors are known to be implicated in autoaggregation of *Escherichia coli*, such as Antigen 43 (Ag43), the product of the *flu* gene (Diderichsen, 1980; Hasman *et al.*, 1999), curli (Olsén *et al.*, 1989), the AAF/I and AAF/II aggregative adherence fimbriae produced by enteroaggregative *E. coli* (EAEC) (Nataro *et al.*, 1992; Czeczulin *et al.*, 1997) and the bundle-forming pili (BFP) produced by enteropathogenic *E. coli* (EPEC) (Bieber *et al.*, 1998).

We are particularly interested in type 1 fimbriae, which are rigid 7-nm-wide and ≈ 1-μm-long, rod-shaped surface structures found on the majority of *E. coli* strains and widespread among other members of the Enterobacteriaceae (Klemm and Krogfelt, 1994). A typical type 1 fimbriated bacterium has 200–500 peritrichously arranged fimbriae on its surface. Interaction between type 1 fimbriae and receptor structures has been shown in a number of studies to play a key role in the colonization of various host tissues by *E. coli* (Yamamoto *et al.*, 1990; Bloch *et al.*, 1992) and in biofilm formation on abiotic surfaces (Pratt and Kolter, 1998; Schembri and Klemm, 2001a). Also, in certain strain backgrounds, type 1 fimbriae can be regarded as virulence factors. Indeed, we and others have shown previously that the expression of type 1 fimbriae in *E. coli* is linked to urinary tract pathogenesis (Connell *et al.*, 1996; Mulvey *et al.*, 1998; Sokurenko *et al.*, 1998).

Type 1 fimbriae are heteropolymers; the bulk of the structure is made up of about 1000 copies of the major subunit protein, FimA, polymerized into a right-handed helical structure. Additionally, small quantities of the minor components FimF, FimG and FimH are also present (Klemm and Christiansen, 1987; Krogfelt and Klemm, 1988). It has been shown that the receptor-recognizing element of type 1 fimbriae is the 30 kDa FimH protein (Krogfelt *et al.*, 1990). The FimH protein is located at the tip and possibly also interspersed along the fimbrial shaft (Abraham *et al.*, 1987; Krogfelt *et al.*, 1990; Jones *et al.*, 1995). The FimF and FimG components are probably required for integration of the FimH adhesin into the fimbrial organelle (Klemm and Christiansen, 1987; Jones *et al.*, 1995).

By virtue of the FimH adhesin, type 1 fimbriae mediate adhesion to a variety of mannosylated glycoproteins. Additionally, FimH-mediated binding to protein targets such as laminin (Kukkonen *et al.*, 1993), collagen (Pouttu *et al.*, 1999) and fibronectin (Sokurenko *et al.*, 1994; Schembri *et al.*, 2000) as well as abiotic surfaces (Pratt and Kolter, 1998; Schembri and Klemm, 2001a) has been reported. Changes in receptor specificity were found to result from minor variations in the amino acid sequence of FimH. Also, the affinity of FimH towards mannose targets can vary as a result of changes in its primary structure. In about 80% of faecal *E. coli* isolates, the FimH adhesin is

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only capable of binding to trimannose receptors. In contrast, the FimH adhesins from ≈70% of urinary tract isolates carry minor mutations (compared with the faecal isolates), which enhance the ability to recognize monomannose receptors (Sokurenko *et al.*, 1995). The mutant alleles confer a significantly higher tropism for the uroepithelium (Sokurenko *et al.*, 1998). A number of structure–function studies have indicated that the N-terminal half of FimH is involved in receptor recognition (Schembri *et al.*, 1996; Langerman *et al.*, 1997; Knudsen and Klemm, 1998; Sokurenko *et al.*, 2001). This was confirmed when the three-dimensional structure of the FimC–FimH complex was elucidated (Choudhury *et al.*, 1999). According to this, the FimH protein is folded into two domains, an N-terminal carbohydrate-binding domain (residues 1–156) linked by a short tetrapeptide loop to a C-terminal organelle integration domain (residues 160–279), with anchorage of FimH to the bulk of the organelle being provided via donor strand complementation. In a previous study, we described the construction of a FimH mutant library by polymerase chain reaction (PCR)-induced random mutagenesis of the *fimH* gene (Schembri *et al.*, 2000). Specific mutations were identified that altered the ability of FimH to bind to monomannose, oligomannose and protein targets. In this work, we report on novel FimH variants that mediate autoaggregation of bacteria.

Results

Isolation of autoaggregating clones

The mature FimH protein consists of 279 amino acid residues. A FimH mutant library was created consisting of a pool of *fimH* genes with PCR-introduced random mutations within the segment encoding amino acids 10–225 of the mature FimH protein of *E. coli* K-12 (Schembri *et al.*, 2000). In order to express the FimH variants as constituents of fimbriae, a helper plasmid, pPKL115, encoding all *fim* genes except *fimH*, was used for transcomplementation of the *fimH*-encoding plasmids. During routine growth of liquid aliquots of the mutant library, small fast-forming precipitates of cells were sometimes observed. When such precipitates were carefully sampled and grown as liquid cultures, a large fraction of the entire culture was seen to settle when the tubes were left standing. This behaviour is not seen in the case of wild-type FimH. In order to select further for aggregating mutants, the procedure was repeated three times, after which the precipitate was plated for single colonies.

Fifty of the enriched clones were randomly selected and tested for their ability to aggregate from standing liquid cultures. Twenty-two of the 50 clones were observed to autoaggregate and settle rapidly under these conditions. To ensure that the observed aggregation phenotype was indeed the result of specific alteration of the *fimH* gene, each of the *fimH*-encoding plasmids was isolated and retransformed into S1918(pPKL115). The new recombinant clones displayed the same aggregation phenotype as the original isolates, indicating that the phenotype was indeed plasmid encoded. The FimH mutants all conferred rapid settling of cells from standing liquid cultures (Fig. 1), identical to the settling phenotype mediated by the Ag43 autoaggregation protein. We refer to this phenotype as FimH-mediated autoaggregation.

Sequence characterization of fimH *clones that induce aggregation*

The nucleotide sequences of the *fimH* genes from the 22 aggregating clones were determined. The sequences revealed that there were indeed only three different clone types; the three plasmids were referred to as pMAS54, pMAS60 and pMAS62. Plasmid pMAS54 was found in 13 of the 22 clones (59%), plasmid pMAS60 was found in six of the clones (27%), and plasmid pMAS64 was present in three clones (14%). The *fimH* variants all contained multiple codon changes, which were all located in a region corresponding to the first 155 amino acids of the mature FimH protein, i.e. within the lectin domain of FimH

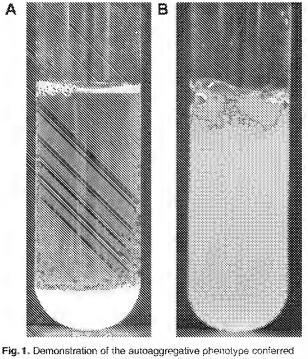


Fig. 1. Demonstration of the autoaggregative phenotype conferred by one of the FimH variants selected during the enrichment procedure (A) compared with that of cells producing normal type 1 fimbriae (B). Cells were grown overnight with shaking at 37°C and allowed to settle for 4 h.

(Table 2). Plasmids pMAS54 and pMAS60 had nine and six changes, respectively, that caused amino acid alterations in FimH. Plasmid pMAS62 had three changes that resulted in amino acid alterations in FimH and, additionally, a frameshift mutation in codon position 92 (Table 1).

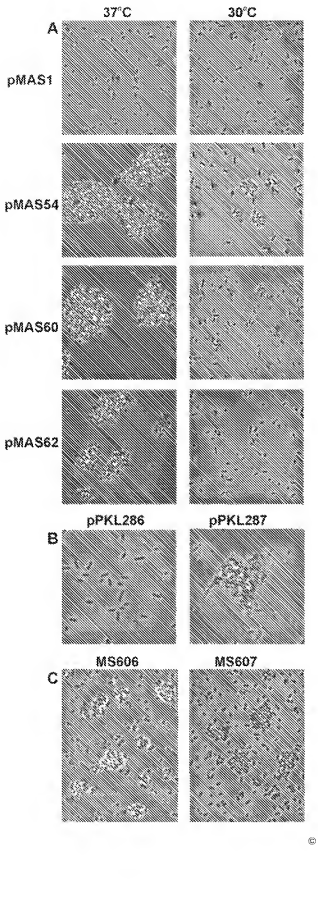
FimH-mediated autoaggregation phenotype

Bacterial autoaggregation is a phenomenon often associated with colonization of host surfaces and can be readily assessed by light microscopy. Indeed, examination of cells from each of the three clones by light microscopy revealed the presence of large, tightly packed aggregates of cells (Fig. 2). These aggregates were unable to be dispersed by vortexing or by the addition of high concentrations of methyl-α-D-mannopyranoside (αmm), a known inhibitor of FimH-mediated agglutination of yeast cells. Also, growth in the presence of αmm did not inhibit autoaggregation. Intriguingly, the FimH-mediated autoaggregative phenotype of all three clones was temperature dependent; at 30°C, the cells did not form prominent aggregates after

Table 1. Strains and plasmids used in this study.

Strain/plasmid	Relevant genotype	Reference/source
E. coli strains		
HB101	F′ *laqI*^{q}	Boyer and Roulland-Dussoix (1969)
HEHA16	Δ*fim* Δ*flu* derivative of BD1302	This study
MS72	pMAS1 and pPKL115 in *S1918*	This study
MS105	Δ*fimH* derivative of ORN194	This study
MS170	pMAS54 and pPKL115 in S1918	This study
MS202	pMAS60 and pPKL115 in S1918	This study
MS204	pMAS62 and pPKL115 in S1918	This study
MS211	pMAS69 and pPKL115 in S1918	This study
MS589	pKEN2 in S1918	This study
MS590	pKEN2 and pHHA13 in S1918	This study
MS606	*fimH* plasmid from Cl#4 in S1918(pPKL114)	This study
MS607	*fimH* plasmid from MJ2-2 in S1918(pPKL114)	This study
ORN194	IPTG *fim*-inducible strain	Woodall *et al.* (1993)
PK799	pPKL236 and pPKL115 in S1918	This study
PK800	pPKL238 and pPKL115 in S1918	This study
PK802	pPKL237 and pPKL115 in S1918	This study
PK803	pPKL239 and pPKL115 in S1918	This study
PK920	pPKL286 and pPKL114 in S1918	This study
PK921	pPKL287 and pPKL114 in S1918	This study
PK922	pPKL288 and pPKL114 in S1918	This study
PK923	pPKL289 and pPKL114 in S1918	This study
PK925	pPKL286 in MS105	This study
PK926	pPKL287 in MS105	This study
PK927	pPKL288 in MS105	This study
PK928	pPKL289 in MS105	This study
S1918	F′ *laqI*^{q} Δ*fimB–H::kan*	Brown (1992)
Plasmids		
pKEN2	Constitutively expressed *gfp* gene in pBR322	Cormack *et al.* (1996)
pHHA13	Wild-type *fim* gene cluster in pACYC184	H. Hasman
pPKL4	Wild-type *fim* gene cluster in pBR322	Klemm *et al.* (1985)
pPKL114	All *fim* genes except fimH in pBR322	Klemm *et al.* (1994)
pPKL115	All fim genes except fimH in pACYC184	Pallesen *et al.* (1995)
pMAS1	*fimH* gene in pUC19	Schembri and Klemm (1998)
pMAS35	*fimH* deletion plasmid	This study
pMAS54	Modified *fimH* gene (V28A, V56A, F71L, Y82H, T87A, V94A, W103R, S113G, V118A)	This study
pMAS60	Modified *fimH* gene (V30L, G73E, S114R, N136Y, Q143L, V155G)	This study
pMAS62	Modified *fimH* gene (S80R, W103R, V145A; Δ92[a])	This study
pMAS69	Modified *fimH* gene (Δ89[a])	This study
pMW119	pSC101-based low-copy-number cloning vector	Nippon Gene
pPKL236	Modified *fimH* gene (W103R, S113G, V118A)	This study
pPKL237	Modified *fimH* gene (V28A, V56A, F71L, Y82H, T87A, V94A)	This study
pPKL238	Modified *fimH* gene (S114R, N136Y, Q143L, V155G)	This study
pPKL239	Modified fimH gene (V30L, G73E)	This study
pPKL286	*fimH* from pMAS1 in pMW119	This study
pPKL287	fimH from pMAS54 in pMW119	This study
pPKL288	*fimH* from pMAS60 in pMW119	This study
pPKL289	*fimH* from pMAS62 in pMW119	This study

a. Deletion of one base at these codon positions of the mature FimH protein.



overnight growth in LB broth, whereas at 37°C, the opposite was observed (Fig. 2). It should be noted that, in the case of the pMAS54 FimH, small-cell aggregates were still observed at 30°C. This temperature-dependent autoaggregation phenotype was corroborated by examination of the settling profiles of the clones when grown at either 30°C or 37°C (Fig. 3). In line with the microscopic observations described above, only the pMAS54 FimH mutant could promote flocculation of cells at 30°C, albeit to a lesser degree than that observed at 37°C. Taken together, our data indicate that autoaggregation is FimH based and temperature dependant.

Autoaggregation is not the result of high-copy-number effects

The *fimH* variants and auxiliary *fim* genes used were located on the high-copy-number vectors pUC19 and pACYC184 respectively. In order to eliminate any hypothetical effects of the high-copy scenario, the *fimH* genes were subcloned into the pMW119 low-copy vector, which is based on the pSC101 plasmid with a copy number of ≈ 3 (Lobner-Olesen, 1999). Additionally, we constructed an *E. coli* K-12 strain, MS105, which is a *fimH* derivative of ORN194. This strain has chromosomally located *fim* genes, the expression of which is driven by a *lac* promoter. Upon induction with IPTG, MS105 hosts harbouring plasmids pPKL287, pPKL288 or pPKL289, but not the wild-type control (pPKL286), were observed to autoaggregate. The autoaggregation conferred by one of these plasmids, pPKL287, is illustrated in Fig. 2B.

The mutant FimH proteins are displayed on the cell surface

In wild-type fimbriae, FimH is displayed on the bacterial surface as an integral component of the fimbrial organelle. The classical way of monitoring type 1 fimbriae-mediated adhesion to eukaryotic cells is agglutination of erythrocytes or yeast cells. Yeast cell agglutination is the most conserved binding property among natural *E. coli* isolates

Fig. 2. A. Phase-contrast microscopy demonstrating the autoaggregative phenotype of cells expressing FimH variants encoded on plasmids pMAS54, pMAS60 and pMAS62. As a control, the phenotype of cells expressing the *E. coli* K-12 FimH (pMAS1) is also shown. The effect of temperature on FimH-mediated bacterial autoaggregation was assessed by growth at both 30°C and 37°C.
B. Phase-contrast microscopy demonstrating the autoaggregation phenotype invoked by the low-copy-number FimH-expressing plasmids pPKL286 (control *E. coli* K-12 FimH) and pPKL287 (FimH variant originating from pMAS54).
C. Phase-contrast microscopy demonstrating the autoaggregative phenotype of *E. coli* S1918(pPKL114) cells expressing FimH variants from wild-type UTI strains CI#4 (MS606) and MJ2-2 (MS607).

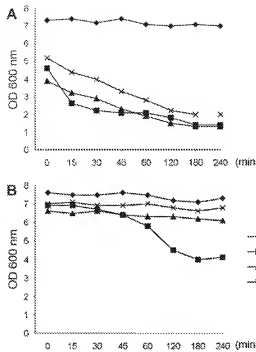


Fig. 3. Settling profile of the FimH autoaggregating variants. Cells were grown in LB broth overnight at 37°C (A) or 30°C (B) with shaking. At the beginning of the experiment, all cultures were vortexed vigorously for 10 s and, at regular time intervals, 100 µl samples were taken from the top of the tube and the OD_{600} was measured.

and is specifically mediated by FimH. To demonstrate surface display of the mutant FimH proteins, we tested the ability of the strains to agglutinate yeast cells. Only the pMAS54 and pMAS62 FimH proteins were able to cause agglutination, albeit not as efficiently as the wild-type FimH, and this agglutination was inhibitable by αmm. In the case of the pMAS62 FimH, yeast agglutination was somewhat surprising, as it contained a frameshift mutation in codon position 92 (see later). The inability of the pMAS60 FimH to cause yeast cell agglutination indicates that some combination of the S114R, N136Y, Q143L and V155G mutations was responsible for abolishing mannose binding. In this respect, it is important to note that mutagenesis of position 136 in FimH was shown previously to abolish agglutination (Schembri *et al.*, 1996).

In order to demonstrate surface display of the FimH variants further, fimbriae preparations from each of the autoaggregating clones were submitted to Western blotting using a FimH-specific antiserum (Fig. 4). In all cases, clear signals were observed corresponding to FimH. Taken together, our results indicate that the observed changes in FimH do not impede organelle integration and surface display of FimH.

Translational frameshifting alleviates deletions in fimH

The mature form of FimH consists of 279 amino acid residues. The *fimH* mutant encoded by plasmid pMAS62 contains a deletion of one base in codon 92 (corresponding to amino acid 92 in the mature FimH). Arguably, this should result in misreading of the downstream sequence before reaching a stop codon at position 109 and result in a truncated FimH protein. The AGY-decoding $tRNA^{Ser}_{GCU}$ shows a marked propensity to induce translational frameshifting by decoding a doublet codon (Farabaugh, 1996). Interestingly, codon 88 actually is AGC. Accordingly, the AGC codon at position 88, which is prone to $tRNA^{Ser}_{GCU}$-mediated translational frameshifting (causing a shift in the −1 direction), might, in combination with the deletion of one base in codon 92 (causing a shift in the +1 direction), actually reinstate the normal reading frame. The sequence in this region would then be read as ^{88}SRNAAV^{93} instead of the wild-type sequence ^{88}SETPRV^{93}. According to this mechanism, a significant number of translation events would give rise to normal-sized FimH products. To test this hypothesis, we introduced a deletion of one base in codon 89 of the *fimH* gene resulting in plasmid pMAS69. Interestingly, S1918(pPKL115) hosts transformed with plasmid pMAS69 show good yeast agglutination titres (albeit lower than the wild type), indicative of the presence of functional FimH on the cell surface (data not shown). Furthermore, when fimbriae preparations from strains expressing the variant FimH proteins encoded by either pMAS62 or pMAS69 were submitted to Western blotting, using a FimH-specific antiserum, clear signals corresponding to full-length FimH were observed (Fig. 4). As expected, the amounts of FimH protein from both strains were reduced compared with the wild-type control.

FimH-mediated aggregation is independent of Ag43

Ag43, the product of the *flu* gene, is a self-recognizing, surface-located adhesin instrumental in cell aggregation

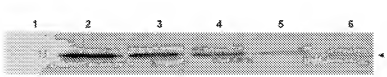


Fig. 4. Western blot of total fimbriae preparations from *E. coli* S1918(pPKL115) (lane 1) and the same strain expressing FimH variants encoded by plasmids pMAS1 (lane 2), pMAS54 (lane 3), pMAS60 (lane 4), pMAS62 (lane 5) and pMAS69 (lane 6). The blot was reacted with anti-FimH serum. The position of the FimH protein is indicated.

and settling as a result of intercellular Ag43–Ag43 interaction (Hasman *et al.*, 1999). Ag43 is the only protein of *E. coli* K-12 known to mediate this phenotype. We have demonstrated previously that expression of type 1 fimbriae abrogates Ag43–Ag43 interaction by physically impeding the close cell–cell contact required for Ag43-mediated autoaggregation (Hasman *et al.*, 1999). Furthermore, the *fim* and *flu* systems cross-talk at the transcriptional level (Schembri and Klemm, 2001b). To rule out any involvement of Ag43 in the present observations, an *E. coli fim, flu* double mutant (HEHA16) was constructed. Upon transformation of this host with our *fimH*-encoding plasmids and the auxiliary plasmid pPKL115, we observed exactly the same autoaggregating phenotypes as seen with the S1918 host. Thus, the FimH-mediated autoaggregation phenotype is independent of Ag43 expression and can be reproduced in different *E. coli* background strains.

FimH-mediated aggregation seems to result from multiple amino acid changes

The FimH-mediated autoaggregative variants identified in this study contained multiple amino acid changes. In an attempt to define the mutations responsible for the autoaggregation phenotypes, we used overlapping PCR to split the mutations (Table 2). These split clones were constructed in such a way that each new clone contained approximately half the original mutations in comparison with their parent FimH-mediated autoaggregative variants. None of the split FimH variants displayed the autoaggregating phenotype in the S1918(pPKL115) host background, indicating that this phenotype results from the concerted action of multiple changes in FimH.

FimH from wild-type strains can induce autoaggregation

The FimH variants isolated after our *in vitro* mutagenesis procedure invoke a novel autoaggregation phenotype not reported previously. A number of FimH variants from wild-type *E. coli* urinary tract infection (UTI) strains have been extensively characterized for their binding affinity to mannose substrates and proteins (Sokurenko *et al.*, 1995). As a result of our observations, we decided to test whether the FimH-mediated autoaggregation phenomenon could in any way be reproduced by wild-type FimH variants. We introduced plasmids expressing wild-type *fimH* variants from the UTI strains CI#4 and MJ2-2 (Sokurenko *et al.*, 1995) into the S1918(pPKL114) background strain. Indeed, upon examination of overnight cultures, these clones exhibited a significant degree of autoaggregation when compared with our K-12 FimH control (Fig. 2C). Although this autoaggregation was not as pronounced as in our mutant clones, the observations indicate that this phenotype may have some relevance to type 1 fimbriae-associated virulence of the urinary tract.

Investigations of the mechanism underlying FimH-induced autoaggregation

The FimH-mediated autoaggregative phenotype may be the result of various alternative intercellular interactions. To examine whether the mutant FimH adhesins bound specifically to other cell surface components, we tagged potential target cells with green fluorescent protein (GFP). Neither *E. coli* S1918 (Δ*fim* strain expressing GFP; data not shown) nor *E. coli* S1918(pHHA13) (expressing type 1 fimbriae and GFP) was able to bind to any of the autoaggregative clones. This was examined by growing the strains separately and then mixing them or by growing them together as a mixed culture (Fig. 5). Taken together, the results indicate that the autoaggregation mediated by our FimH variants is not the result of binding to other *E. coli* surface components or to other fimbrial structural proteins. We then examined the morphology of the fimbriae produced by our FimH-expressing autoaggregative clones by electron microscopy. Each of the clones, unlike the

Table 2. Summary of FimH-mediated autoaggregation phenotypes.

Plasmid	Amino acid changes in FimH	Autoaggregation[a]	Agglutination[b]
pMAS1	K-12 wild-type FimH	–	+
pMAS54	V28A V56A F71L Y82H[c] T87A V94A W103R S113G V118A	+ +	+
pMAS60	V30L G73E S114R N136Y Q143L V155G	+ +	–
pMAS62	S92R W103R V145A (Δ92[d])	+ +	+
pPKL237	V28A V56A F71L Y82H T87A V94A	–	+
pPKL236	W103R S113G V118A	–	+
pPKL239	V30L G73E	–	+
pPKL238	S114R N136Y Q143L V155G	–	–
CI#4 FimH	V27A N70S G73E S78N T201D	+	+
MJ2-2 FimH	V27A G66D N70S S78N V163A	+	+

a. FimH-mediated autoaggregation.
b. Agglutination of yeast cells.
c. FimH surface-exposed residues are underlined.
d. Deletion of one base in codon 92 of the mature FimH protein.

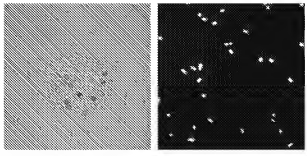


Fig. 5. Phase-contrast microscopy of autoaggregating cells (*E. coli* S1918 containing pMAS54 and pPKL115) mixed with *E. coli* S1918(pHHA13) (expressing both type 1 fimbriae and GFP). No association was observed between the two different cell types, indicating that the FimH-mediated autoaggregation phenotype is not the result of specific binding to other cell surface components. Left, phase-contrast microscopy; right, visualization of cells expressing GFP.

wild-type control, produced twisted and entangled fimbriae that seemed to give rise to a meshwork of intertwined fibres (Fig. 6).

Discussion

Many bacteria have the ability to autoaggregate, resulting in the formation of compact cell clusters or tight communities of cells. In *E. coli*, a number of different mechanisms have been described that promote the spontaneous formation of multicellular clusters. Ag43 is a surface-located autotransporter protein that mediates autoaggregation of cells in static liquid cultures (Owen *et al.*, 1987; Hasman *et al.*, 1999). This phenomenon is mediated by intercellular Ag43–Ag43 interaction and can be visualized directly by light microscopy and settling of standing liquid cultures (Hasman *et al.*, 1999). The expression of Ag43 also results in a characteristic frizzy colony morphology (Hasman *et al.*, 2000). Curli are surface organelles formed on the outside of the cell by the precipitation of secreted soluble subunit proteins into thin fibres (Hammar *et al.*, 1996). Autoaggregation probably results from intercellular fibre precipitation mediated by a nucleator protein that can be secreted by the same cell or adjacent cells. Bundle-forming pili (BFP) are a type IV class of fimbriae produced by EPEC strains that emanate from the cell surface and align along their longitudinal axes to form bundles of filaments (Giron *et al.*, 1991; Bieber *et al.*, 1998). These fimbriae are long, flexible, rope-like structures composed almost exclusively of a single repeating structural subunit referred to as BfpA (Giron *et al.*, 1991). Expression of BFP mediates two phenotypes thought to play a role in colonization: autoaggregation in liquid cultures and localized adherence on tissue culture cell monolayers (Bieber *et al.*, 1998). In EAEC strains, two fimbrial types designated aggregative adherence fimbriae I and II (AAF/I and AAF/II) have been identified (Nataro *et al.*, 1992; Czeczulin *et al.*, 1997). These flexible 2- to 3-nm-wide structures are thought to be members of the Dr family of adhesins (Nataro and Kaper, 1998). The aggregative adherence phenotype is distinguished by prominent autoagglutination of bacterial cells to each other (Nataro *et al.*, 1987).

Type 1 fimbriated *E. coli* are able to recognize mannose and protein targets (Sokurenko *et al.*, 1994; 1997).

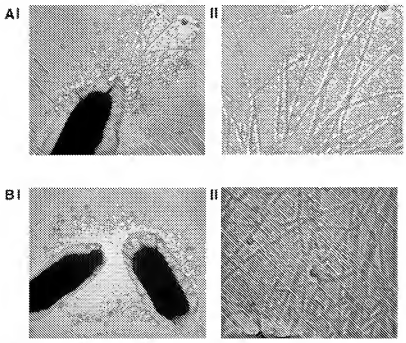


Fig. 6. A. Electron microscopy of control strain expressing wild-type FimH (I) and close-up view of the corresponding fimbriae (II).
B. Electron microscopy of autoaggregating cells (I) and close-up view of the corresponding fimbriae indicating the nature of the convoluted organelle structure (II).

A number of studies have attempted to define the integral parts of the FimH adhesin that contribute to receptor recognition. Linker insertion mutagenesis of FimH in positions corresponding to amino acids 56 and 136 in the mature protein completely abolished binding to D-mannose receptors (Schembri *et al.*, 1996). Minor structural variations occurring naturally in the FimH adhesin of *E. coli* type 1 fimbriae that can lead to physiologically important changes in the pattern of receptor recognition have also been found within the N-terminal half of the FimH protein (Sokurenko *et al.*, 1994; Pouttu *et al.*, 1999; Schembri *et al.*, 2000). Two reports using fusions of sectors of FimH with either MalE or FocH indicated a segment encompassing amino acid residues 3–158 as constituting a core region for receptor recognition, with additional information residing in the 159–201 region (Thankavel *et al.*, 1997; Knudsen and Klemm, 1998). In this study, we have identified a novel FimH-mediated autoaggregative phenotype from our *fimH* mutant library.

The cell aggregates formed by the mutant FimH adhesins were stable and formed in LB broth under shaking conditions. Construction of a *fim, flu* double mutant confirmed that this phenotype was independent of Ag43 expression. The phenotype displayed by our *fimH* mutant strains shares some similarity to the *E. coli* autoaggregation mechanisms described above. In each case, prominent cell aggregates form in liquid culture medium that can be viewed directly by light microscopy. However, a major difference in the autoaggregative phenotype observed here is that the cell clusters could not be dispersed by physical shear forces. Indeed, this phenotype is somewhat similar to a BFP mutant strain reported to form static multicellular aggregates that could not be dispersed (Bieber *et al.*, 1998). A similar autoaggregative phenotype has also been attributed to the expression of thin aggregative fimbriae in some *Salmonella* species (Collinson *et al.*, 1993; Römling *et al.*, 2000). It is remarkable that minor modifications in FimH can create fimbriae with novel and dramatically altered functional properties. Our data underline the functional flexibility of the FimH adhesin. It is highly likely that the autoaggregating ability of some FimH variants is linked to biofilm formation in hydrodynamic environments, e.g. the urinary tract (Schembri and Klemm, 2001a). This is not very surprising, given that other autoaggregating substances such as Ag43 and curli are also associated with biofilm formation (Kjærgaard *et al.*, 2000; Prigent-Combaret *et al.*, 2000).

In a previous study, a temperature-sensitive *fimH* mutant was described that could agglutinate guinea pig erythrocytes after being grown at 42°C but not at 31°C (Harris *et al.*, 1990). This prompted us to investigate whether the FimH-mediated autoaggregation phenotype could be altered by growth temperature. Indeed, we observed a striking difference in cell aggregation at 30°C and 37°C. The exact reasons for this are unclear; however, we propose that some FimH variants may be susceptible to temperature-dependent conformational changes that alter their functional properties. Further studies are required to elucidate the molecular mechanisms of this phenomenon.

Three different FimH mutants were identified from our random mutant library that induced an autoaggregative phenotype. Each of the mutants contained different amino acid alterations, with only the W103R mutation common to FimH mutants from plasmids pMAS54 and pMAS62. Dissection of these mutants by the construction of split clones indicated that the autoaggregative phenotype of the three FimH mutants is the cumulative effect of multiple amino acid changes (Table 2). Furthermore, it is apparent that very different mutations are able to evoke the same phenotype. This observation is in line with other studies describing mono- and trimannose binding specificities of FimH (Schembri *et al.*, 2000). Although the region encoding amino acids 10–225 of the mature FimH protein was mutagenized, we only found changes within the 156-amino-acid lectin domain of the FimH adhesin. The FimH variants of wild-type origin (Cl#4 and MJ2-2) each had five changes, two of which were identical (Table 2). A similar pattern is observed for these variants, i.e. alterations occur primarily in the lectin domain, and only one residue outside this domain is altered. Based on the three-dimensional structure of FimH (Choudhury *et al.*, 1999), we looked at the localization of the alterations with respect to surface exposure. A significant proportion of the changes was indeed surface exposed (Table 2), but no obvious pattern was evident. With respect to the library-derived variants, it is interesting to note that the ability of two of the autoaggregating clones to agglutinate yeast cells (the third did not) was sensitive to αmm, although αmm had no effect on autoaggregation. This might indicate that the autoaggregation is distinct from the mannose recognition of FimH. Our data suggest that FimH-mediated autoaggregation is not the result of intercellular binding to some cell surface component, including type 1 fimbriae. However, the present autoaggregating strains expressed fimbriae with a twisted and curved appearance not seen in the wild-type control. It might be that the aggregation phenomenon is caused by some form of intercellular fimbrial entanglement. The localization of the FimH adhesin in the fimbriae is somewhat controversial. FimH has been demonstrated to be present on the tip of the organelle as an integral part of a short fibril structure (Jones *et al.*, 1995). Meanwhile, other studies have additionally suggested it to be interspersed along the fimbrial shaft (Abraham *et al.*, 1987; Krogfelt *et al.*, 1990). It is tempting to believe that the mutant forms of FimH described here can somehow influence fimbrial morphology. Although the

present mutations are located in the part of *fimH* that encodes the receptor-binding domain, the full extent of FimH sectors that are involved in organelle biogenesis and subunit–subunit interaction are not known at present and, in principle, the reported mutations could affect such parameters.

Our emerging view of FimH depicts it as a multifaceted protein prone to microevolution. Minor changes in this adhesin have been shown to promote a pathogenicity adaptive phenotype that is associated with enhanced virulence of the mouse urinary tract (Sokurenko *et al.*, 1998). From our mutant library, we have isolated FimH variants that induce autoaggregation of *E. coli*. This phenotype has not been identified before and could in principle be a laboratory curiosity. It was therefore comforting to note that wild-type FimH variants originating from uropathogenic isolates are able to confer a similar autoaggregating phenotype. Furthermore, in the mouse UTI model, type 1 fimbriae-expressing *E. coli* have been observed to form microcolonies (Connell *et al.*, 2000) and, thus, one might predict that some degree of autoaggregation may be associated with type 1 fimbriae-associated virulence properties.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table 1. The *E. coli* K-12 strain HB101 (F′ *lacI kan*) (Boyer and Roulland-Dussoix, 1969) was used as an intermediate host during plasmid construction. All subsequent phenotypic analyses were performed in the *E. coli* Δ*fim* strain S1918 (Brown, 1992), HEHA16, ORN194 or MS105. HEHA16 is a Δ*fim* Δ*flu* derivative of *E. coli* BD1302 (Diderichsen, 1980) and was constructed using the temperature-sensitive gene replacement plasmid system described previously (Kjærgaard *et al.*, 2000). ORN194 contains a chromosomally located *fim* locus fused to the *lacUV5* promoter and can be readily induced by IPTG (Woodall *et al.*, 1993). MS105 is a *fimH* deletion mutant of strain ORN194 and was constructed essentially as described previously (Schembri *et al.*, 1996). To facilitate the construction of this strain, the kanamycin resistance gene from the *fimH* deletion plasmid pCH103 was substituted with an erythromycin resistance gene cassette. This plasmid was referred to as pMAS35. The FimH expression vector pMAS1 contains the *fimH* gene from *E. coli* K-12 strain PC31 (Klemm *et al.*, 1985) under the transcriptional control of the *lac* promoter (Schembri and Klemm, 1998). In addition, the plasmid contains unique *Kpn*I and *Hin*dIII recognition sequences within the *fimH* gene, which flank the region encoding the proposed FimH receptor-binding domain (Fig. 1). Plasmids pPKL114 and pPKL115 contain all the *fim* genes except *fimH* in either pBR322 or pACYC184 respectively (Klemm *et al.*, 1994; Pallesen *et al.*, 1995). Plasmid pMW119, a derivative of pSC101, is a low-copy-number (≈ three copies per cell) cloning vector (Nippon Gene). Cells were grown in Luria–Bertani (LB) broth (Sambrook *et al.*, 1989) supplemented with the appropriate antibiotics.

DNA techniques

Isolation of plasmid DNA was carried out using the QIAprep Spin plasmid kit (Qiagen). Restriction endonucleases were used according to the manufacturer's specifications (Biolabs or Pharmacia). The nucleotide sequences were determined on both DNA strands by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). Oligonucleotide primers were purchased from Gibco BRL.

Construction of the fimH *mutant library*

The construction of the *fimH* mutant library has been described previously (Schembri *et al.*, 2000). Briefly, the 650 bp *Kpn*I–*Hin*dIII fragment of the *fimH* gene from pMAS1 was mutagenized by nucleotide misincorporation during suboptimal PCR conditions. Four reactions were performed, which contained three of the four nucleotides at a concentration of 50 mM and the other at 5 mM respectively. Each reaction contained 7 mM $MgCl_2$ to increase the stability of non-complementary basepairs and 0.5 mM $MnCl_2$ to reduce the template specificity of the polymerase. The error-prone PCR procedure was performed for 35 cycles with two primers (ms7 and ms8) that flank the *Kpn*I and *Hin*dIII sites of the *fimH* gene. The amplification products were combined, digested with *Kpn*I and *Hin*cII, purified after agarose gel electrophoresis and religated into similarly cut plasmid pMAS1 to construct a library of altered *fimH* genes. To permit expression of the corresponding FimH variants as functional constituents of type 1 fimbriae, the ligation mix was transformed into *E. coli* strain S1918 (Δ*fim*) containing an auxiliary plasmid, pPKL115, which encodes the entire *fim* gene cluster except *fimH*. The transformation mixture was made up to 10 ml, grown to approximately 10 times the initial library diversity and stored as aliquots at −80°C in 25% (v/v) glycerol. Analysis of 300 random transformants revealed that the mutagenesis procedure was highly successful, with ≈ 60% of transformants displaying an altered yeast agglutination phenotype.

Autoaggregation screening assay

An aliquot of the *fimH* mutant library was grown overnight in LB, diluted to ≈ 1×10^8 cells ml^{-1} and allowed to settle without agitation for 4 h. A small volume of the culture was then removed from the bottom of the tube and grown overnight in the same manner. This procedure was repeated a further four times, after which the cultures were streaked for single colonies. Fifty clones were then selected and tested individually for their ability to mediate bacterial autoaggregation, as evidenced by rapid settling of cells after overnight growth in LB broth at 37°C under shaking conditions.

Construction of defined fimH *mutations*

Specific amino acid substitutions from the mutant *fimH* genes were introduced into the wild-type *fimH* sequence by overlapping PCR. The following primers were used: ms1,

5′-GTGATAAGCTTCACCATACCTACAGC (upstream *fimH* primer); ms2, 5′-GCTCGAATTCCAGCATTAGCAATGTCC (downstream *fimH* primer); 137, 5′-ATAATCGAGAACGGAT AAGC; 138, 5′-GCTTATCCGTTCTCGAATTAT (overlapping internal *fimH* primers). Each construct was sequenced to ensure fidelity of the PCR reaction. Plasmids containing these chimeric *fimH* genes were introduced into S1918(pPKL115) and tested for autoaggregation and yeast cell agglutination.

Agglutination of yeast cells

The capacity of bacteria to express a D-mannose-binding phenotype was assayed by their ability to agglutinate yeast (*Saccharomyces cerevisiae*) cells on glass slides. Aliquots of washed bacterial suspensions at $OD_{550} = 0.5$ and 5% yeast cells were mixed, and the time until agglutination occurred was measured. Furthermore, clones that did not cause any agglutination under these conditions were also tested at $OD_{550} = 20$ and/or low temperature, but still did not react.

Autoaggregation assays

Settling profiles were performed on overnight cultures (10 ml) grown at both 30°C and 37°C. At the beginning of the experiment, all cultures were shaken vigorously for 10 s. Samples (100 μl) were taken from the top of the culture at regular time intervals, and the optical density was measured at 600 nm (OD_{600}).

To examine for an effect of αmm on FimH-mediated autoaggregation, strains were grown overnight in the presence of 1% αmm. We also examined whether the same concentration of αmm could disperse existing bacterial aggregates upon addition to overnight cultures.

GFP constructs

Plasmid pKEN2 encoding constitutive expression of GFP was transformed into a *fim*-negative (S1918) or *fim*-positive [S1918(pHHA13)] background. GFP-expressing cells were mixed with FimH-mediated autoaggregating cells lacking GFP, and the resultant cell mixture was observed by using a Carl Zeiss Axioplan epifluorescence microscope equipped with a filter for detecting GFP.

Western blotting

Fimbriae were prepared as described previously (Klemm *et al.*, 1998). Samples were subjected to SDS–PAGE, blotted onto polyvinylidene difluoride (PVDF) filters and treated as described previously (Stentebjerg-Olesen *et al.*, 1997). A polyclonal anti-FimH rabbit serum (a kind gift from E. Sokurenko, University of Washington, Seattle, USA) was used as the primary serum, and peroxidase-conjugated anti-rabbit serum was used as the secondary serum.

Microscopy

Electron microscopy was carried out essentially as described previously (Klemm *et al.*, 1994). In short, a 10 μl aliquot of bacterial suspension was placed on a carbon-coated, glow-discharged grid for 30 s. Grids were washed in two drops of PBS, dehydrated in increasing concentrations of ethanol, blotted dry and shadowed with tungsten. Cells were viewed with a Jeol 100B electron microscope.

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